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PRINCIPAL INVESTIGATOR: Cindy A. Wilson

CONTRACTING ORGANIZATION: University of California
Los Angeles, CA 90024

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Cindy A. Wilson, Gina Bernardo, Hong Mei Rong,
Judy Dering, Charles Ginther and Dennis Slamon

E-Mail: ciwilson@amgen.com

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14. ABSTRACT

Our objective was to determine if signaling through the ROR1 receptor tyrosine kinase controls the proliferation, self-renewal and/or differentiation of mammary epithelial cells. We silenced ROR1 by stable expression of siRNA ROR1 sequences using a retroviral expression system in human cell lines with progenitor properties. We also overexpressed the ROR1 cDNA in immortalized human mammary epithelial cell lines (184A1, 184B5). The effects of depleted and overexpressed ROR1 were assayed using *in vitro* matrigel TDLU formation assays and in vivo xenograft tumor formation assays. We characterized the CAL51 breast cancer line extensively because of its high level of ROR1 expression and its interesting multi-potent properties. When we silenced ROR1 in this cell line we observed compelling differences in the formation of *in vitro* 3D matrigel structures and also in the histology and nuclear morphology of the resulting xenograft tumors. Expression profiling experiments comparing CAL51 tumor xenografts with and without ROR1 depletion uncovered a potential role for ROR1 in controlling the developmental fate of breast cells. These experiments led us to hypothesize that ROR1 antagonizes the Hedgehog pathway that is thought to play a critical role in the maintenance of adult stem cells.

15. SUBJECT TERMS
ROR1, mammary progenitor cells, differentiation, Wnt pathway, Hedgehog pathway

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Introduction

Human estrogen receptor (ER) positive tumors and mouse mammary tumors induced by oncogenic Neu or H-Ras express cell type markers consistent with a differentiated luminal origin (e.g. cytokeratins K8/K18). In contrast, aggressive ER-negative human cancers and murine tumors induced by the Wnt-1 oncogene, display a much more heterogeneous pattern of cell type markers including the basal cytokeratins K5, K17, K14, and stem cell antigen (1). This is consistent with the idea that multi-potent progenitor cells are the targets of transformation in these breast cancers. Immortalized progenitor cells capable of differentiating into both luminal and myoepithelial lineages have been described (2,3). Although many commonly studied human breast cancer cell lines express homogeneous luminal markers, we recently identified multiple malignant breast cell lines that may have progenitor properties since they produce heterogeneous cell populations in terms of luminal and basal markers. We found that both non-malignant and cancer lines with progenitor properties consistently express the ROR1 receptor tyrosine kinase while luminal mammary cells have no detectable expression. Furthermore, the ROR receptor family (ROR1/2) have been reported to bind Wnt ligands via its extracellular frizzled domain thus providing a link to Wnt signaling which has an established role in progenitor cell self-renewal (4,5). Our objective is to determine if signaling through the ROR1 receptor tyrosine kinase controls the proliferation, self-renewal and/or differentiation of mammary progenitor cells. Our approach is to silence ROR1 by stable expression siRNA ROR1 sequences and to overexpress ROR1 constructs (wild type, constitutively activated and deletion mutants) using retroviral systems in human and mouse mammary progenitor cells and cancer cell lines. The effect of depleted and overexpressed ROR1 is assayed using *in vitro* matrigel TDLU formation assays and xenograft tumor formation assays in combination with microarray expression profiling.

Body

Differentiation markers characteristic of multiple cell types in the mammary gland have emerged as a dominant feature in gene expression profiles that segregate primary human breast cancers. A large number of breast cancer cell lines have been isolated and individually characterized over the last few decades. We, and others, have begun to comprehensively align these cell lines with primary tumors based on gene expression profiles and other parameters in order to improve the relevance of data obtained from these experimental models for understanding human disease. We have extensively characterized a large panel of 51 breast cell lines by expression profiling as well as their growth patterns in plastic, in 3-D matrigel culture and in vivo as tumor xenografts. Baseline gene expression profiles were generated for each of 51 breast cell lines grown under normal, sub-confluent conditions using Agilent human V1 oligonucleotide chips. The labeled cRNA for each cell line was competitively hybridized to a mixed reference RNA comprised of 10 breast cell lines selected to achieve high variability in several well known breast cancer marker genes. A total of 46 malignant breast cell lines were profiled along with a human mammary epithelial cells (HMEC) culture and three immortalized HMEC cell lines. Principle component analysis (PCA) was performed using all differentially regulated genes in the 51 cell line profiles (Fig 1A). This analysis resulted in two main cell line populations separated by the PC2 (y axis) consistent with a luminal class (cyan and blue) and a non-luminal class (red, green and orange) based on previously published classification analysis of primary breast tumors. Agglomerative hierarchical clustering using the same set of all differentially expressed genes resulted in a dendogram with a similar, high-level distinction between luminal and non-luminal cell types (Fig 1B). Both unsupervised analyses yielded the same 25 luminal and 26 non-luminal cell lines.

We corroborated the expression of several of the key differentiation markers at the level of protein expression by immunofluorescent (IF) staining (Fig. 2). The IF analysis correlates well with the microarray data and provides additional information on cytoskeletal protein expression at the level of individual cells. The luminal cell lines showed strong homogeneous staining for KRT8/18 and/or KRT19 (i.e. MCF-7 and KPL-1). The mesenchymal cell lines had very little keratin expression and stained uniformly and strongly positive for vimentin (i.e. HCC1395). Many of the remaining non-luminal cell lines showed a mixture of staining patterns comprised of weak and/or heterogeneous luminal keratin expression, or basal KRT14 expression or a mixture of the two (i.e. 184A1, MCF10A, CAL51). The MDA-MB-231 cell line has a clear mesenchymal marker profile and predominant vimentin staining, however it also contains a small but stable population of large cells that are strongly KRT19 positive. The CAL51 cell line was unique among the malignant cell lines in that it also contains a small fraction of α-smooth muscle actin positive cells. It was also unusual in that it clustered with the mesenchymal cell lines in the unsupervised analysis even though it contains relatively low levels of vimentin.

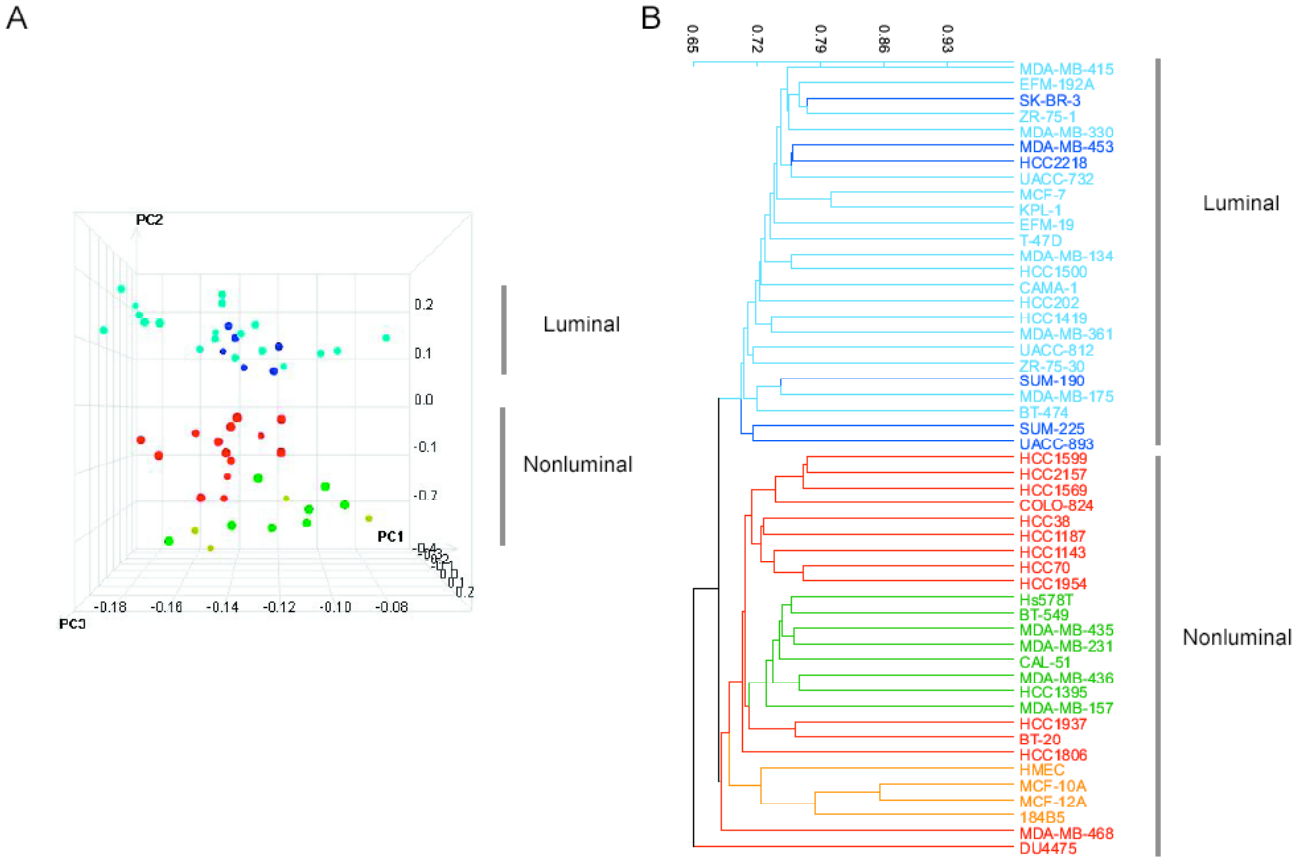


Figure 1. Luminal differentiation is a dominant feature of breast cancer cell line expression profiles. The expression profile for each cell line was generated on the Agilent Human 1A (22K) oligonucleotide arrays using a mixture of 10 breast cell lines with highly variable properties as the reference. All cell lines are derived from malignant primary breast cancers or metastasis except the HMEC, 184B5, MCF10A and MCF12A lines, which are normal mammary epithelial cells or immortalized derivatives. **A)** Principle Component Analysis (PCA) and **B**) hierarchical clustering of the 51 breast cell lines was performed using all 7177 differentially regulated genes (> 2 fold change with p values < 0.01 in 3 or more experiments).

We find ROR1 to be expressed exclusively in non-luminal cell lines. The highest expressing cell line was the basal HCC1187 followed by the mesenchymal CAL51 line. The average level of ROR1 in the "over-expressing" cell lines compared to the average expression in luminal breast cancer cell lines is 43 fold higher. The range is 120 fold higher in the case of HCC1187 (highest) and 15 fold higher on the lower end (BT-20). Immortalized, non-malignant breast lines appear to be of basal/progenitor origin and they also express ROR1 at levels significantly higher than luminal breast cancer cells (orange points; Figure 3b). The level of ROR1 expression, however, is higher in the basal breast *cancer* compared to non-malignant basal cells: (i.e. the average of the 12 ROR1 positive cancer cell lines compared to the average of

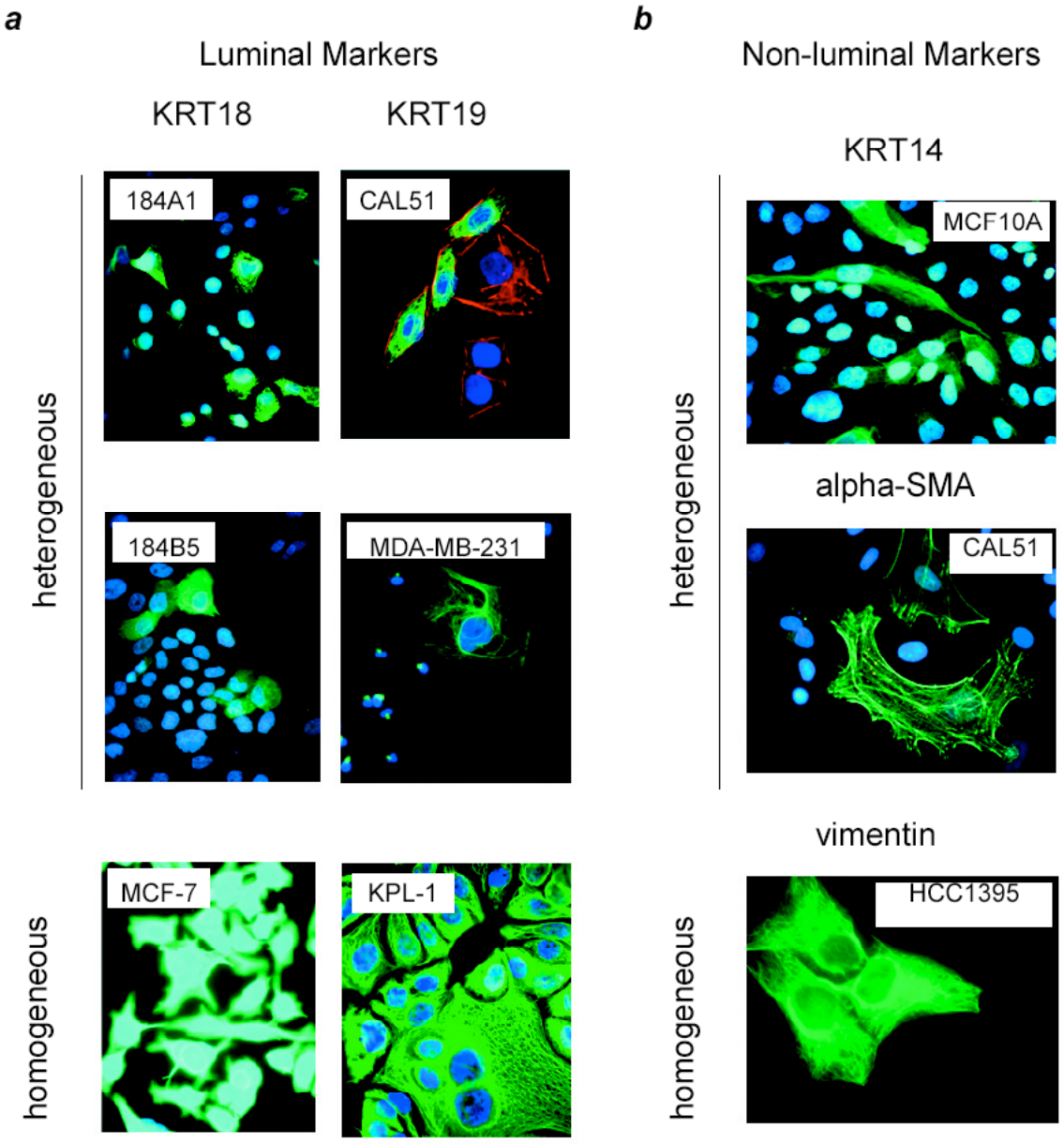
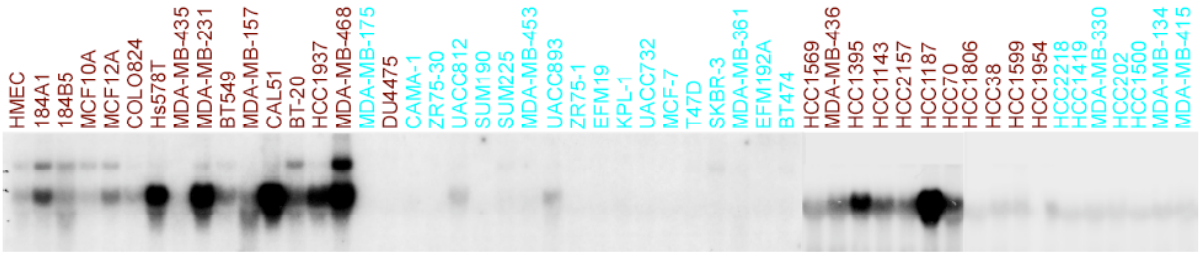


Figure 2. The non-luminal cells often display heterogeneous staining for differentiation markers whereas the luminal cell lines generally express homogeneous simple glandular cytokeratins (KRT8/18). ***(a)*** Examples of basal/progenitor and mesenchymal cells stained with the luminal markers KRT8/18 and KRT19. These cells (184A1, 184B5, CAL51 and MDA-MB-231) show low level heterogeneous staining for these two markers. The luminal cell lines MCF-7 and KPL1 express high levels of KRT8/18 and KRT19 in every cell. The MCF-7 image was photographed at the same setting as the 184A1 & 184B5 pictures for comparison. ***(b)*** Examples of cells stained with non-luminal markers. The basal/progenitor/mesenchymal cells can express heterogeneous patterns of KRT14 and alpha-SMA (smooth muscle actin) that are typical of the myoepithelial cells in the normal breast. Most of the mesenchymal lines such as HCC1395 express vimentin in every cell and rarely have detectable KRT expression. The exception is the CAL51 cells that have a mixed phenotype.

the 4 immortalized (non-malignant) lines is 7 fold higher). There are no continuously growing non-malignant luminal cells available but based on our analysis of luminal breast cancer and normal tissues, we assume that the expression of ROR1 in *normal* luminal mammary cells is very low or undetectable.

a



b

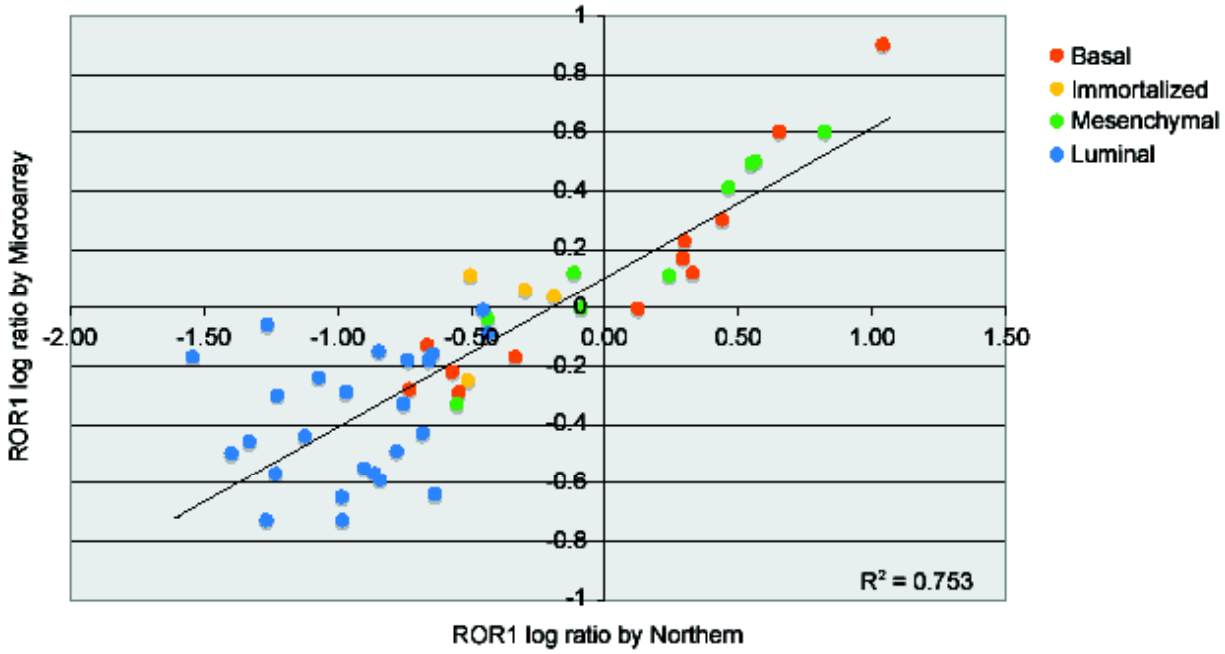


Figure 3. ROR1 is overexpressed in non-luminal breast cancer cell lines. The non-luminal cell lines are divided into three groups: basal, immortalized and mesenchymal. ***(a)*** Northern blots of ROR1 in 51 breast cell lines. ***(b)*** Log ratios for ROR1 in the 51 breast cancer cell lines were obtained by two independent methods. The x-axis values were generated by phosphorimager quantitation of the Northern blots for each cell line compared to the average value of ROR1 for the 10 cell lines used in mixed reference RNA pool in the microarray experiments. The log ratios for the microarray experiments on the y-axis were obtained by direct hybridization of each cell line RNA to the mixed cell line reference.

We have focused our efforts on characterizing the effects of ROR1 silencing in the highest expressing ROR1 cell lines including HCC1187, CAL51, MDA-MB-468, Hs578T, MDA-MB-231, HCC1395 and MCF12A. The CAL51 cells are especially interesting because they appear to have the potential to differentiate into multiple lineages expressing KRT19, smooth muscle actin (Figure 3) and at least 4 stable populations of cells when analyzed on the basis of the differentiation markers CD24 and CD44 (Figure 4). In addition, these cells have been shown to have normal diploid chromosomes and very few amplifications or deletions by array CGH. We have therefore done the most analysis on this multi-potent cell line.

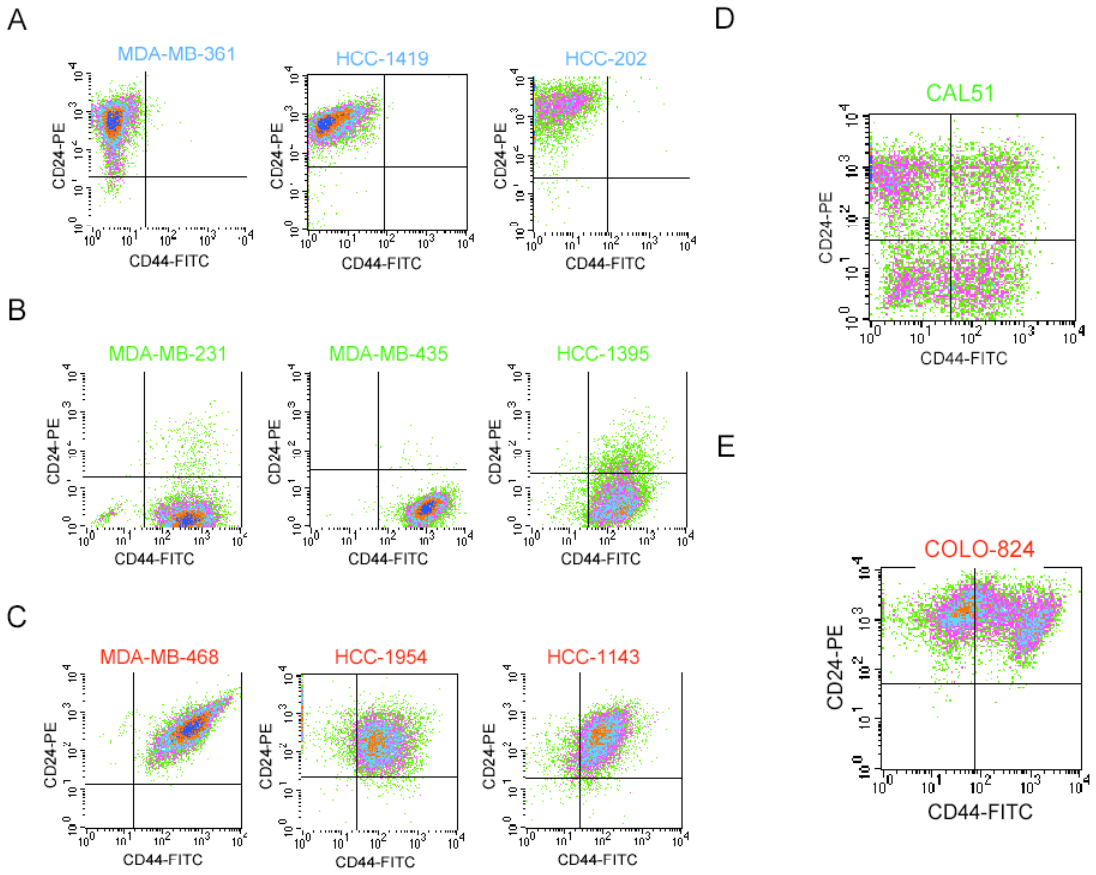


Figure 4. Luminal and non-luminal cell lines have distinct CD24/CD44 expression profiles. (A-E) Representative flow cytometric analysis using labeled CD24 and CD44 antibodies on live cells. A) Luminal cells have a predominant CD24 only phenotype, B) mesenchymal cells have a predominant CD44 only phenotype, C) basal cell have a CD24/CD44 dual phenotype. D) CAL51 cells have at least 4 distinct populations and E) COLO-824 have at least two populations.

Task 1. Generate retroviral expression constructs to express ROR1 and to express small interfering RNA's to silence ROR1:

We designed several 60mer oligonucleotides against ROR1 and cloned them into the pSIREN-retroQ vectors along with a negative control (scrambled sequence) and a luciferase targeting sequence to generate hpRNA expression vectors. We designed 4 of these constructs (Figure 5) and chose the best two (#9 and #16) based on their efficiency of knocking down the expression of ROR1 by transient transfection along with a ROR1 expression plasmid.

	Sequence	Base Pair Position	GC %
#6	AAGTCCAGGATACTCAGATGA	843	43%
#9	AAGTGTTCTCAGTTCGCCATT	1027	43%
#16	AAGTCTGATCTGTGTGACATC	1520	43%
#28	AAGTCCTTGCTGCCCATTCGC	2325	57%

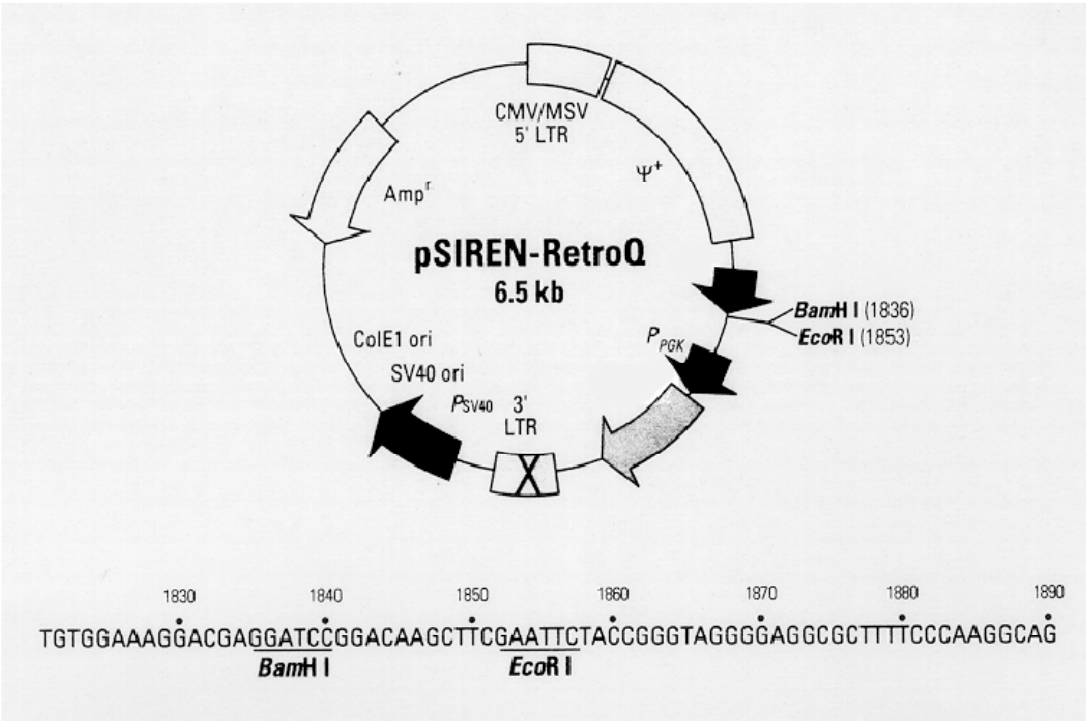


Figure 5. Stable ROR1 silencing vectors. A) Oligonucleotide sequences designed from the ROR1 cDNA sequenced that were cloned into the B) pSIREN-RetroQ expression plasmid.

We also cloned the full length WT ROR1 sequence into an inducible mammalian expression vector (pcDNA4-TO from the TREX system). In addition we made four different point mutations in the cytoplasmic domain (Figure 6). Two of the mutations K506A and D615A) are in the tyrosine kinase domain and are predicted to generate kinase dead mutants. The other two Y722F and Y828A are predicted to disrupt protein-protein interaction sites (See Table 1 in Reportable Outcomes section for complete list of plasmids generated).

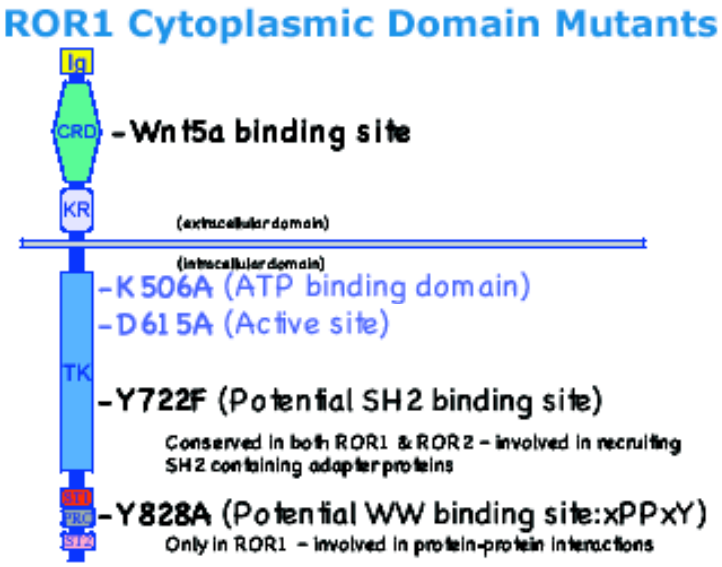


Figure 6. Illustration of point mutations created in the ROR1 cytoplasmic domain.

Task 2. Generate high titer virus producing cell lines:

The two best silencing plasmids (#9 and #16) were transfected along with two negative control plasmids into the NIH3T3 PT76 viral packaging cell line. We then selected cultures with puromycin to isolate individual virus producing clones. We then titered the supernatants from individual clones and chose the highest titer clone for each of the silencing plasmids.

Task 3. Generate stable mammary epithelial cell lines expressing ROR1 silencing constructs and ROR1 expression constructs.

We infected several human mammary epithelial cells including non-malignant cells (i.e. MCF12A) as well as human breast cancer cell lines with luminal and progenitor properties (i.e. CAL51) with ROR1 silencing vectors (See Table 2 in Reportable Outcomes section for complete list of cell lines generated with stable hairpin knock-down of ROR1 message). These cultures were selected with puromycin to isolate individual colonies and in some cases the cultures were

mass selected to generate populations of cells to avoid changes in growth properties to clonal selection and variation. The level of ROR1 was then screened by flow cytometry and immunofluorescence on live cells and by IP-western blotting for the expression of ROR1 protein (Figure 7).

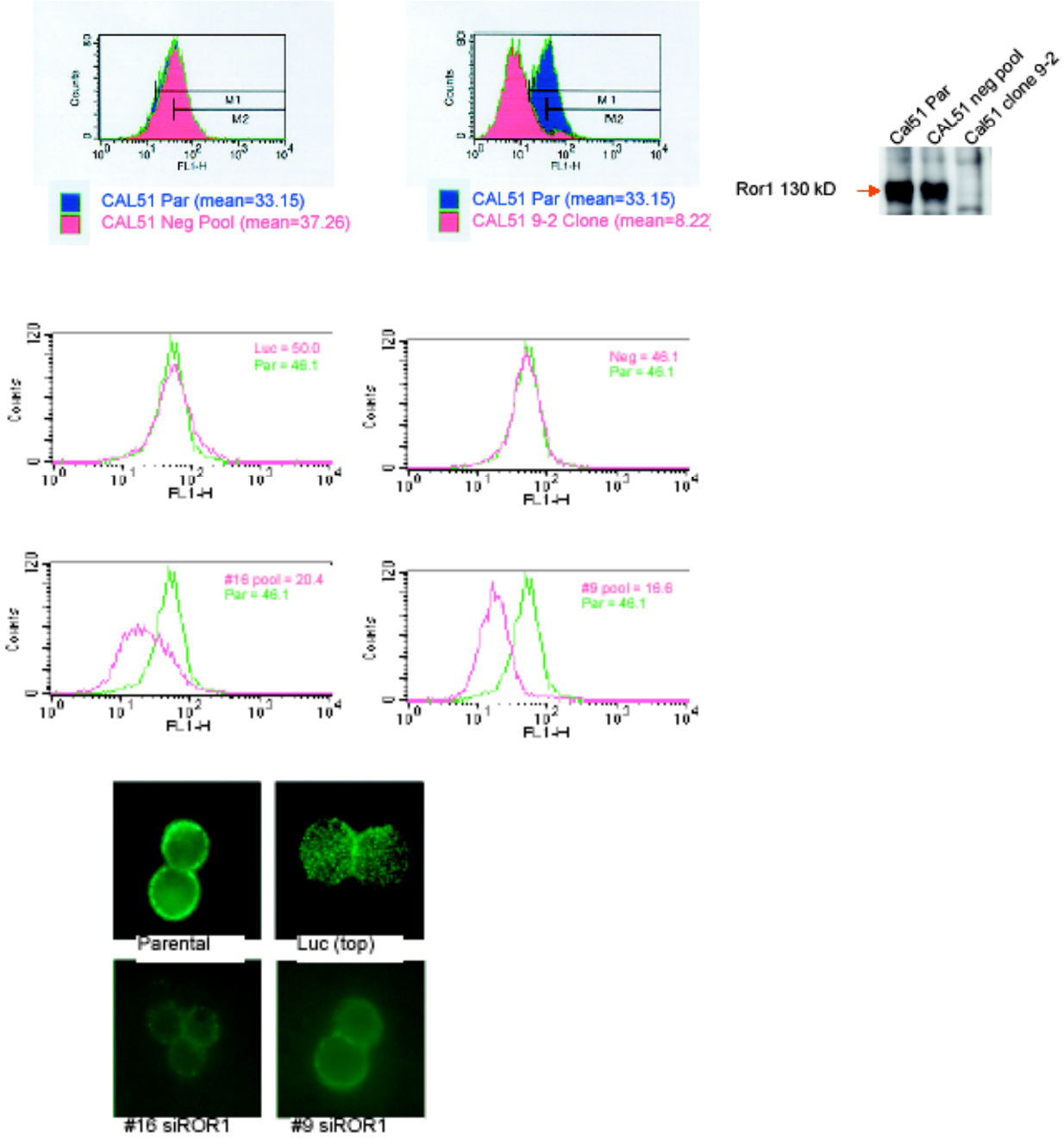


Figure 7. Stable silencing of ROR1 in breast cancer cell lines. A) Flow cytometric analysis of ROR1 expression using a affinity purified rabbit polyclonal antibody. B) IP-western blot of the ROR1 protein shows nearly complete knockdown of expression in CAL51 clone 9-2. C) Flow cytometric analysis of ROR1 expression in the MDA-MB-468 cell lines (green) versus mass selected populations of cells infected with the negative control, luciferase siRNA, #9 ROR1 siRNA or the #16 ROR1 siRNA. D) Immunostaining of live MDA-MB-462 mass selected population with the ROR1 polyclonal antibody.

Task 4. Assay the effects of ROR1 silencing and ROR1 overexpression in mammary progenitor cells and malignant cell lines. (Months 8-12).

We next tested the stable siROR1 cell lines for any changes in proliferation rates that were associated with silencing of ROR1 expression. Although there were some minor changes in growth rates in some of the selected clones it was not always correlated with loss of ROR1 since certain of the selected clones from the negative controls were also changed from the parental cell lines. The mass selected populations did not have proliferation rates significantly different from the parental cells. We conclude that although drug selection and clonal variation can effect proliferation rates, we did not see a see any significant change in proliferation rates of cells grown in 2D on plastic associated with silencing of ROR1

We did however, see a reproducible phenotypic change in the siROR1 cell lines when compared to the parental cells or the negative control siRNA cell lines when the cells were grown in 3D matrigel cultures. The CAL51 parental cell line form structures in which two populations of cell types sort into an inner dense cell mass and outer, more translucent layer. This compartmentalization is lost in the ROR1 siRNA lines a result that may suggest an inability to differentiate into two cell lineages (Figure 8a).

We also grew the various CAL51 cells with and without ROR1 negative control silencing constructs as tumor xenografts in nude mice. We again saw a reproducible histological difference in the resulting tumors by H&E staining (Figure 8b). Loss of ROR1 expression is associated with a loss of tubule formation and a change in nuclear morphology in the CAL51 siROR1 (#9 and #16) xenografts compared to the parental CAL51 or negative control tumors. Again this suggests a loss of an ability to differentiate, and thus a higher-nuclear grade, less-differentiated tumor is produced when ROR1 expression is silenced.

In an effort to corroborate these phenotypic changes, we isolated RNA from these tumors and profiled the gene expression changes associated with loss of ROR1 expression. The schematic for the design of this expression profiling experiment is shown below (Figure 9). RNA from the individual CAL51 tumors from the siROR1 groups were pooled and hybridized to control CAL51 tumor RNA. Representative intensity plots are shown to illustrate the high level of reproducibility of the dye-reversal labeling controls and the pattern of differential gene expression observed in the ROR1 silenced CAL51 tumors compared to the control CAL51 tumors (Figure 10).

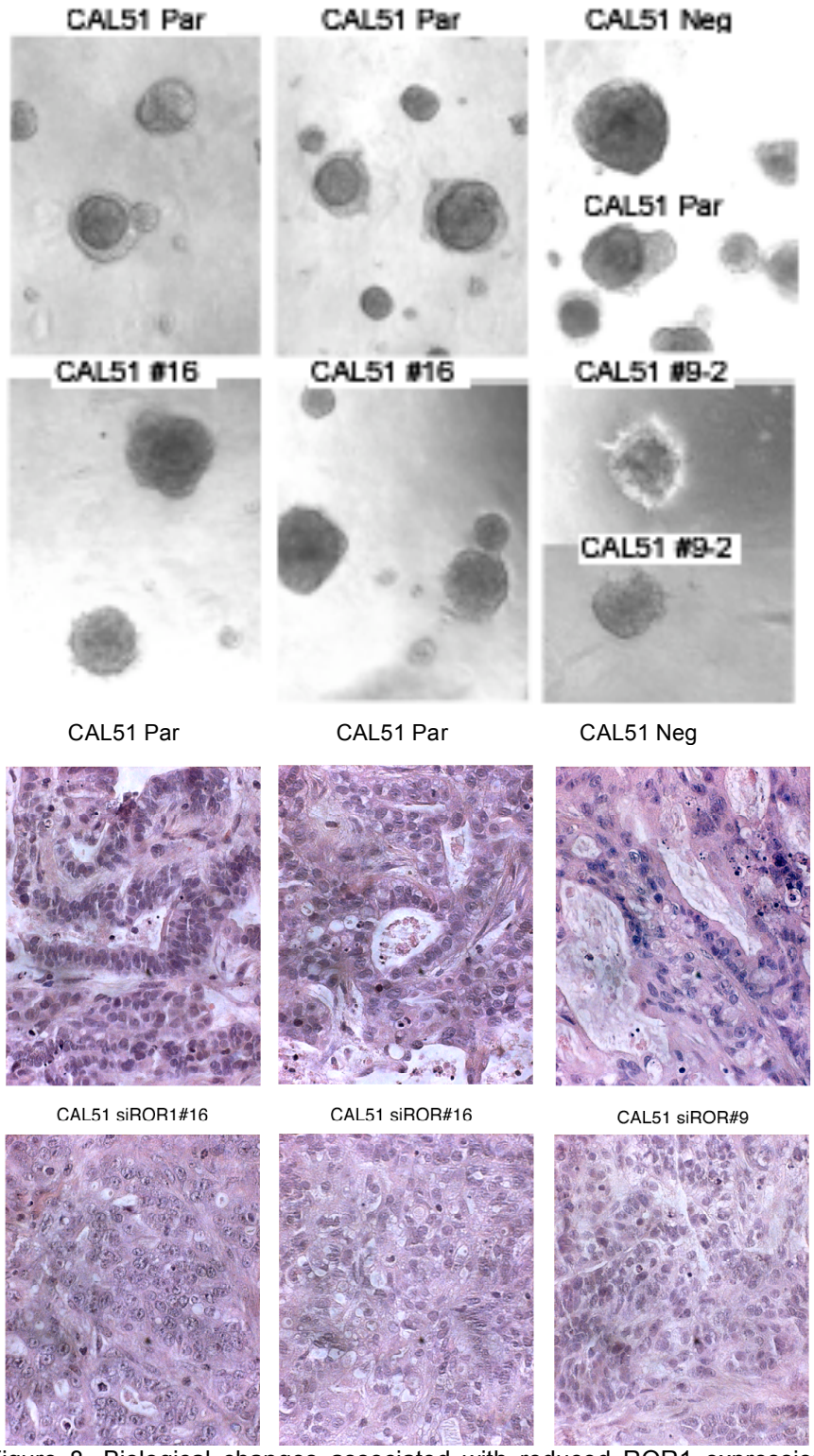


Figure 8. Biological changes associated with reduced ROR1 expression in CAL51 breast cancer cells. A) Loss of ROR1 expression in is associated with altered growth patterns in 3D-matrigel cultures. B) Loss of ROR1 expression is associated with a loss of tubule formation and a change in nuclear morphology in CAL51 xenografts. H&E stained sections of paraffin embedded tumors (40X).

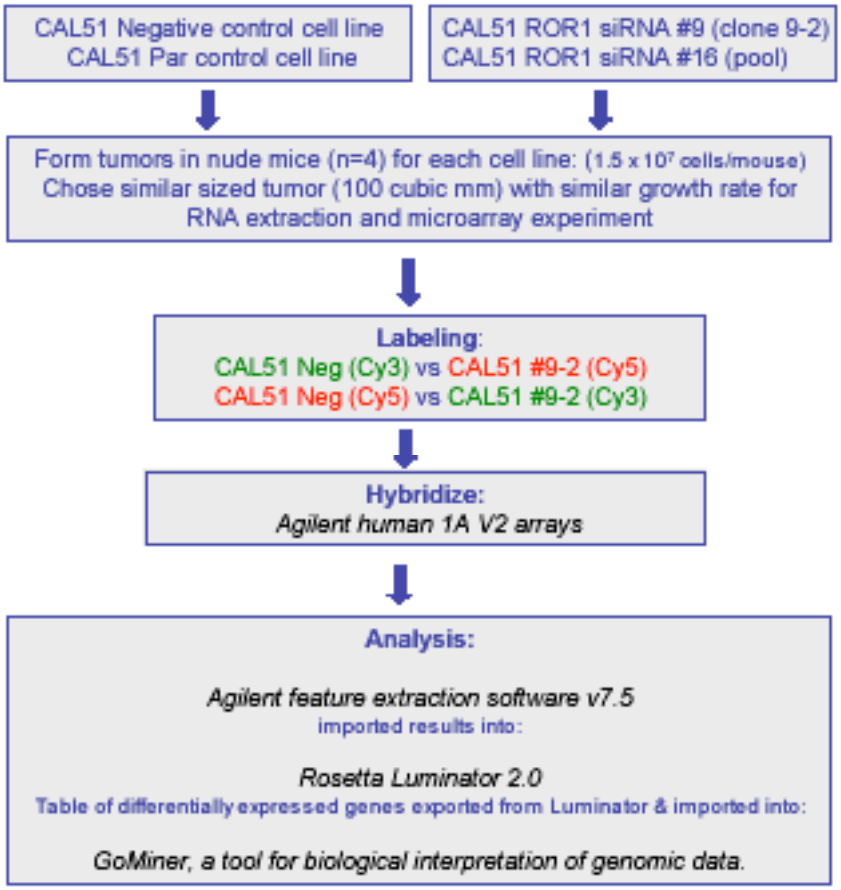
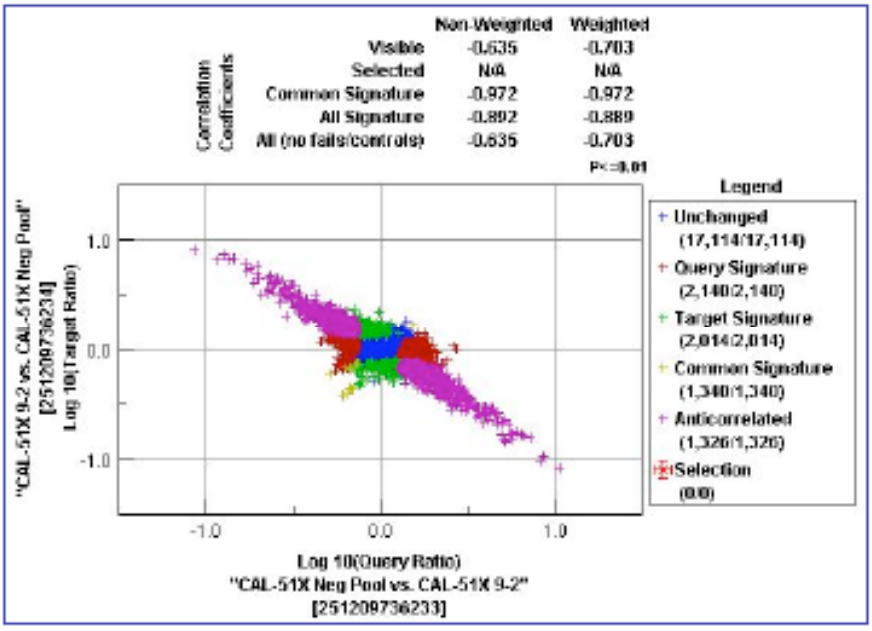


Figure 9. Schematic of the microarray profiling experiment to identify the gene expression changes associated with the loss of ROR1 expression.

We used a combination of analytic tools from Rosetta Luminator and GoMiner to process the differentially regulated genes in the CAL51 siROR1 tumors versus the control tumors. First a gene set consisting of all differentially regulated genes (> 1.5 changed with a p value < 0.01) present in duplicate experiments was derived using the Luminator package. This set of differentially regulated genes then imported into GoMiner and the most significantly changes biological process categories were analyzed further. The Development category showed one of the a large percentages of highly regulated genes (Figures 10 and 11). Within the Development category, the branches of the graph for the Histogenesis pathways were the most affected by ROR1 silencing. The top 40 most significantly down- and up-regulated genes from the Development GoMiner category are listed in below (Figures 12 and 13). Many genes that regulate muscle and bone formation were affected (i.e. myosin, troponin, DKK3, SOSTD1, SPON2, DCN). One of the most interesting findings is that when ROR1 expression is lost we see up-regulation of critical components in the Hedgehog pathway. We observe a 2.3

fold up-regulation of SMO (the hedgehog receptor) and we also see a 2.5 fold up-regulation of IHH (Indian hedgehog; one of the hedgehog ligands). In summary, we have found that depletion of ROR1 in CAL51 breast cells induces changes in growth patterns consistent with a loss of a progenitor cell type and the induction of a developmental genetic program regulating histogenesis (neurogenesis muscle and bone formation).

We also have begun to analyze the phenotype of ROR1 overexpression in non-malignant 184A1 and MCF12A human mammary epithelial cell lines. There appeared to be some selection pressure against expressing high levels in these cells as we never obtained expression levels comparable to the highest levels seen in breast cancer cell lines or the levels we could obtain when we forced expression in a luminal breast cancer cell line such as MCF-7. However, even with a 2-3 fold overexpression of the ROR1 protein in 184A1 and MCF12A cells we observed a striking and highly reproducible phenotype (Figure 14). Both of these parental cell lines grow in very orderly epithelial patterns and form close cell-cell contacts with obvious tight junctions. This pattern remains intact in the vector control cell lines subjected to the same drug selection and cloning as the ROR1 cell lines (Figures 14 and 15). However, this orderly growth pattern is dramatically altered in both cell lines when ROR1 is overexpressed. The cell-cell junctions have been lost and the cells have elongated. The ROR1 cells also show evidence of membrane projections and ruffling characteristic of highly migratory cells. In the future, we hope to better characterize the functional consequences of this phenotype in migration and invasion assay and determine the molecular pathways downstream of ROR1 that are responsible for this phenotype.



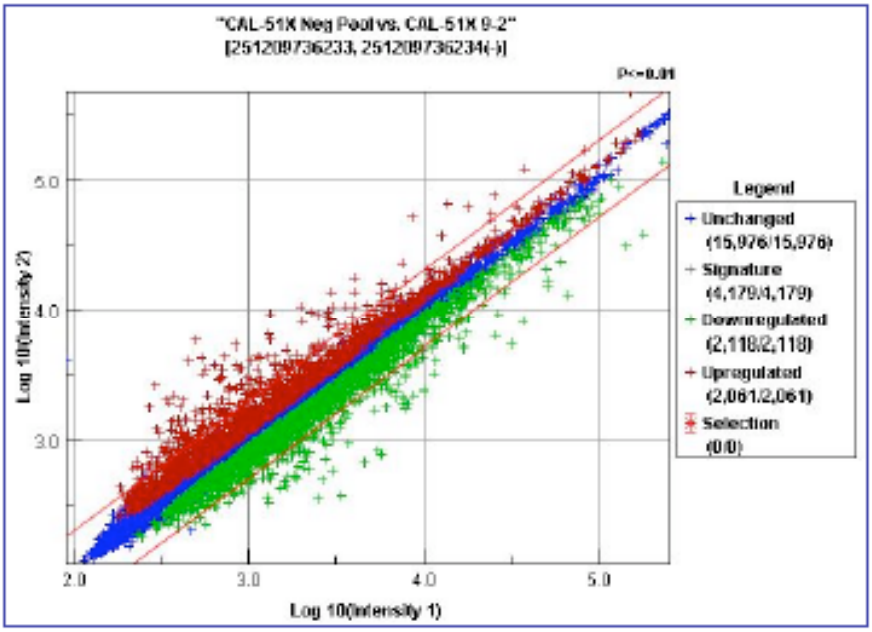


Figure 10. Representative intensity plots for the microarray hybridizations. The top plot is a comparison plot showing the high level of correlation between the two dye-reversal experiments. The bottom intensity plot represents the up- and down-regulated genes after the flour-reversals were combined.

A.

GO ID	Total Genes on Chip	Under	Over	Change	P-Value (Under)	P-Value (Over)	P-Value (Changed)	Term
8150	9478	545	575	1120	0.9997	0.5463	0.9953	biological_process
9987	8555	480	492	972	0.9999	0.9952	1	cellular process
7582	8225	474	484	958	0.9895	0.9285	0.9968	physiological process
50875	7270	413	427	840	0.9913	0.8961	0.9957	cellular physiological process
8152	5421	300	284	584	0.9903	0.9999	1	metabolism
44237	5167	278	265	543	0.998	1	1	cellular metabolism
44238	4965	261	256	517	0.9995	0.9999	1	primary metabolism
7154	2732	158	174	332	0.7725	0.2384	0.4969	cell communication
43170	2486	137	128	265	0.9137	0.9884	0.9956	macromolecule metabolism
44260	2372	127	118	245	0.9551	0.9957	0.9991	cellular macromolecule metabolism
6139	2354	93	109	202	1	0.9997	1	nucleobase, nucleoside, nucleotide and nucleic acid metabol
50789	2353	121	150	271	0.9858	0.2566	0.8572	regulation of biological process
19538	2214	112	115	227	0.9894	0.9779	0.9991	protein metabolism
44267	2200	110	113	223	0.9925	0.9837	0.9995	cellular protein metabolism
7165	2181	127	116	243	0.7143	0.9566	0.9484	signal transduction
50791	2091	106	129	235	0.9862	0.4332	0.9244	regulation of physiological process
51179	1673	87	121	208	0.9537	0.0191	0.3539	localization
51234	1666	87	121	208	0.949	0.017	0.3284	establishment of localization
6810	1665	87	121	208	0.9483	0.0167	0.3248	transport
19222	1635	64	91	155	1	0.8372	0.9999	regulation of metabolism
50874	1570	102	112	214	0.2327	0.0341	0.0284	organismal physiological process
7275	**1560**	**83**	**155**	**238**	**0.9183**	**0**	**0**	**development**
6350	1560	51	85	136	1	0.8797	1	transcription
50896	1549	107	107	214	0.0753	0.0777	0.0169	response to stimulus
19219	1517	51	85	136	1	0.8098	1	regulation_nucleic acid metabolism
45449	1496	48	85	133	1	0.7675	1	regulation of transcription
6351	1483	47	82	129	1	0.8408	1	transcription, DNA-dependent
6355	1444	46	82	128	1	0.7651	1	regulation of transcription, DNA-dependent
43283	1279	51	64	115	0.9998	0.9637	0.9999	biopolymer metabolism
6464	1171	58	50	108	0.9626	0.9982	0.9996	protein modification
9653	1036	62	113	175	0.5642	0	0	morphogenesis
9605	998	77	78	155	0.015	0.0111	0.0005	response to external stimulus
7166	991	46	53	99	0.9819	0.8581	0.9882	cell surface receptor linked signal transduction
7242	849	54	34	88	0.3737	0.9977	0.9573	intracellular signaling cascade
48513	842	50	90	140	0.5845	0	0	organ development
9887	842	50	90	140	0.5845	0	0	organogenesis

B.

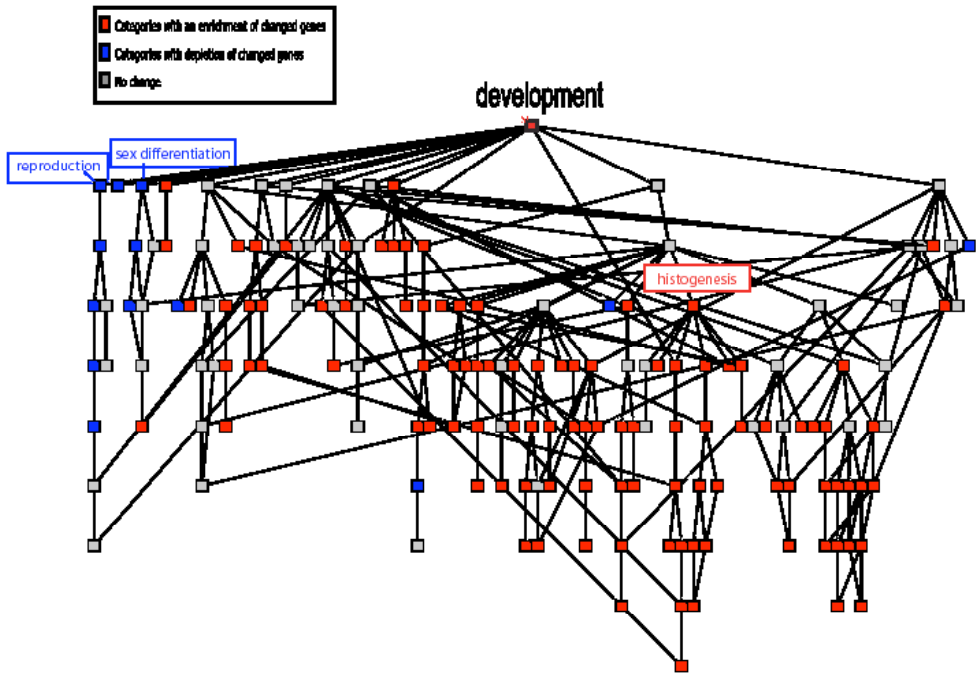


Figure 11. Gene expression changes associated with reduced ROR1 expression in CAL51 breast cancer cells ***A)*** Loss of ROR1 results significant gene expression changes in the "Development" gene ontology (GO) category. An excerpt of the summary table for the biological processes output by GoMiner is shown. **B)** The Directed Acyclic Graph for the Development GO category output by GoMiner.

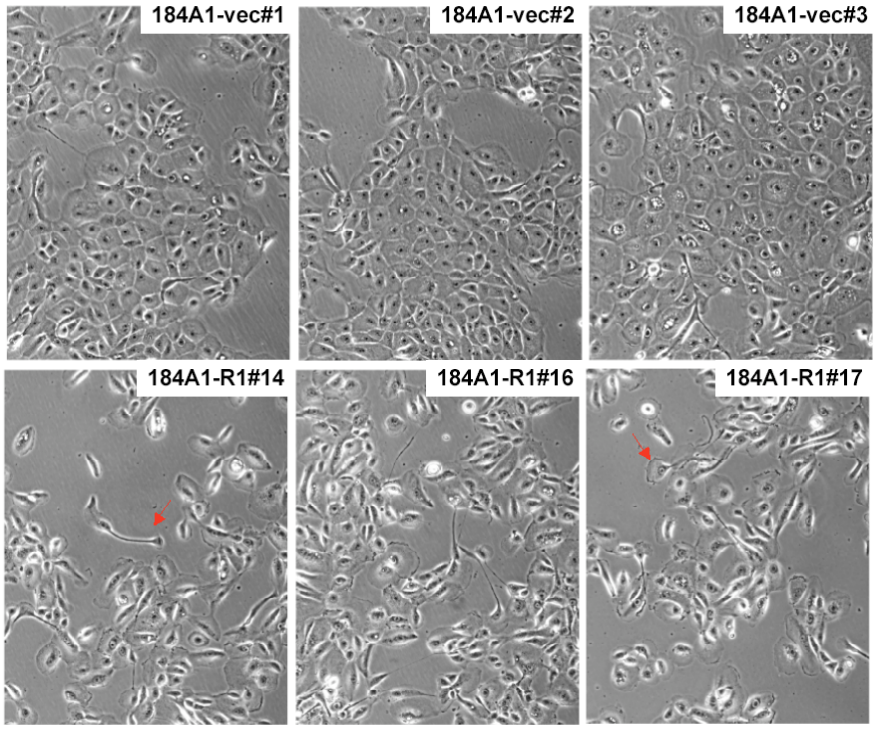
Accession #	Symbol	Name	Fold Change
NM_001432	EREG	Epiregulin	-9.45
NM_005012	ROR1	Receptor tyrosine kinase-like orphan receptor 1	-5.48
NM_015869	PPARG	Peroxisome proliferative activated receptor, gamma	-4.51
NM_001511	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimula	-3.98
NM_002514	NOV	Nephroblastoma overexpressed gene	-3.97
NM_001430	EPAS1	Endothelial PAS domain protein 1	-3.89
NM_017534	MYH2	Myosin, heavy polypeptide 2, skeletal muscle, adult	-3.64
NM_003279	TNNC2	Troponin C2, fast	-3.49
NM_005252	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	-3.10
NM_014164	FXYD5	FXYD domain containing ion transport regulator 5	-3.09
NM_003311	PHLDA2	Pleckstrin homology-like domain, family A, member 2	-2.89
NM_005239	ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-2.43
NM_003282	TNNI2	Troponin I, skeletal, fast	-2.45
NM_017533	MYH4	Myosin, heavy polypeptide 4, skeletal muscle	-2.41
NM_080489	SDCBP2	Syndecan binding protein (syntenin) 2	-2.29
NM_175850	DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	-2.24
NM_003177	SYK	Spleen tyrosine kinase	-2.17
NM_203339	CLU	Clusterin	-2.10
NM_025179	PLXNA2	Plexin A2	-2.03
NM_003290	TPM4	Tropomyosin 4	-2.02
NM_002619	PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-1.93
NM_013441	DSCR1L2	Down syndrome critical region gene 1-like 2	-1.92
NM_201446	EGFL7	EGF-like-domain, multiple 7	-1.91
NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-1.91
NM_001511	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimula	-1.86
BC035854	DSCR1L2	Down syndrome critical region gene 1-like 2	-1.80
NM_002701	POU5F1	POU domain, class 5, transcription factor 1	-1.82
NM_176096	CDK5RAP3	CDK5 regulatory subunit associated protein 3	-1.77
NM_000584	IL8	Interleukin 8	-1.79
NM_006096	NDRG1	N-myc downstream regulated gene 1	-1.77
NM_006080	SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic dom	-1.75
NM_006757	TNNT3	Troponin T3, skeletal, fast	-1.72
AF022375	VEGF	Vascular endothelial growth factor	-1.74
NM_000382	ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	-1.70
NM_002447	MST1R	Macrophage stimulating 1 receptor (c-met-related tyrosine ki	-1.70
NM_005069	SIM2	Single-minded homolog 2 (Drosophila)	-1.69
NM_003379	VIL2	Villin 2 (ezrin)	-1.71
NM_001424	EMP2	Epithelial membrane protein 2	-1.65
NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)	-1.67

Figure 12. Gene expression changes associated with reduced ROR1 expression in CAL51 breast cancer cells. A table of the top 40 most significantly down-regulated genes from the Development category in the CAL51 ROR1 siRNA #9 xenografts compared to control CAL51 xenografts.

Accession #	Symbol	Name	Fold Change
NM_013253	DKK3	Dickkopf homolog 3 (Xenopus laevis)	9.28
NM_001443	FABP1	Fatty acid binding protein 1, liver	9.07
NM_025237	SOST	Sclerosteosis	4.83
NM_000597	IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	4.67
NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	4.21
NM_004484	GPC3	Glypican 3	4.18
NM_002290	LAMA4	Laminin, alpha 4	4.00
NM_001150	ANPEP	Alanyl (membrane) aminopeptidase (aminopeptidase N, ami	3.90
NM_000900	MGP	Matrix Gla protein	3.89
NM_001851	COL9A1	Collagen, type IX, alpha 1	3.87
NM_003483	HMGA2	High mobility group AT-hook 2	3.30
NM_001901	CTGF	Connective tissue growth factor	3.13
NM_003862	FGF18	Fibroblast growth factor 18	3.03
NM_000073	CD3G	CD3G antigen, gamma polypeptide (TiT3 complex)	2.96
NM_000090	COL3A1	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome	2.95
NM_015894	STMN3	Stathmin-like 3	2.91
NM_001920	DCN	Decorin	2.89
NM_005574	LMO2	LIM domain only 2 (rhombotin-like 1)	2.88
NM_012445	SPON2	Spondin 2, extracellular matrix protein	2.80
NM_000522	HOXA13	Homeo box A13	2.79
NM_003633	ENC1	Ectodermal-neural cortex (with BTB-like domain)	2.78
NM_000138	FBN1	Fibrillin 1 (Marfan syndrome)	2.63
NM_015464	SOSTDC1	Sclerostin domain containing 1	2.56
NM_004362	CLGN	Calmegin	2.48
NM_004335	BST2	Bone marrow stromal cell antigen 2	2.47
BC034757	IHH	Indian hedgehog homolog (Drosophila)	2.47
NM_033139	CALD1	Caldesmon 1	2.39
NM_004670	PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	2.37
NM_000582	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialop	2.37
NM_001453	FOXC1	Forkhead box C1	2.35
NM_005631	SMO	Smoothened homolog (Drosophila)	2.26
NM_000088	COL1A1	Collagen, type I, alpha 1	2.17
NM_021080	DAB1	Disabled homolog 1 (Drosophila)	2.14
NM_005982	SIX1	Sine oculis homeobox homolog 1 (Drosophila)	2.14
NM_001321	CSRP2	Cysteine and glycine-rich protein 2	2.13
NM_003462	DNALI1	Dynein, axonemal, light intermediate polypeptide 1	2.12
NM_004787	SLIT2	Slit homolog 2 (Drosophila)	2.08
NM_016307	PRRX2	Paired related homeobox 2	2.05
NM_030582	COL18A1	Collagen, type XVIII, alpha 1	2.04
NM_000304	PMP22	Peripheral myelin protein 22	2.02

Figure 13. Gene expression changes associated with reduced ROR1 expression in CAL51 breast cancer cells. A table of the top 40 most significantly up-regulated genes from the Development category in the CAL51 ROR1 siRNA #9 xenograft compared to the control CAL51 xenografts.

A.



B.

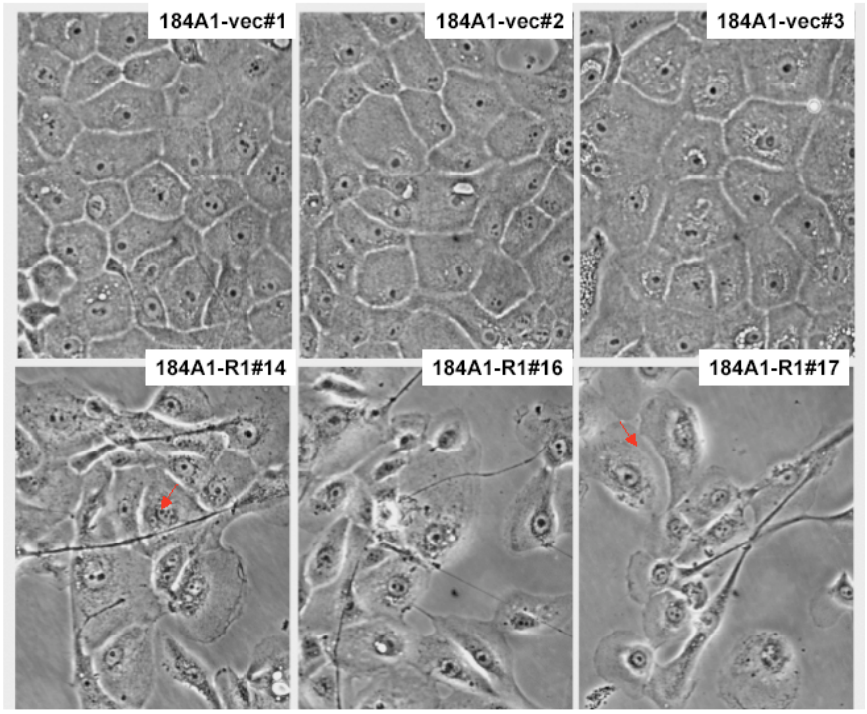
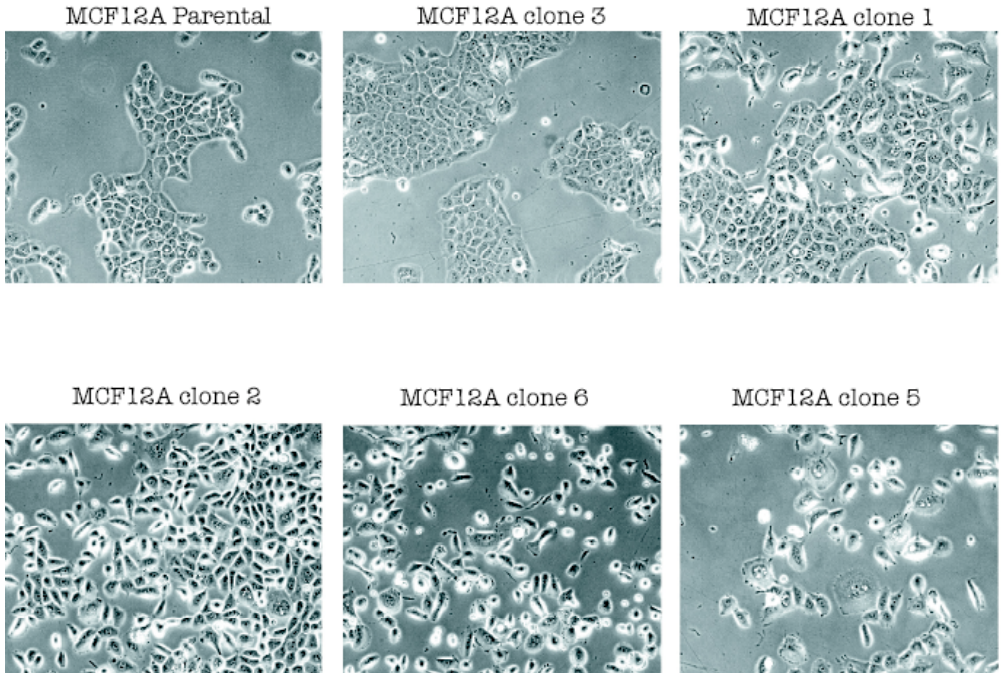


Figure 14. Effects of forced WT ROR1 overexpression on the morphology of immortalized human mammary epithelial cells. 184A1 were transfected with either ROR1 or a vector control, cultures were selected with Zeocin and individual colonies were isolated and expanded. A) Bright field images at 10X. B) Bright field images at 32X.

A.



B.

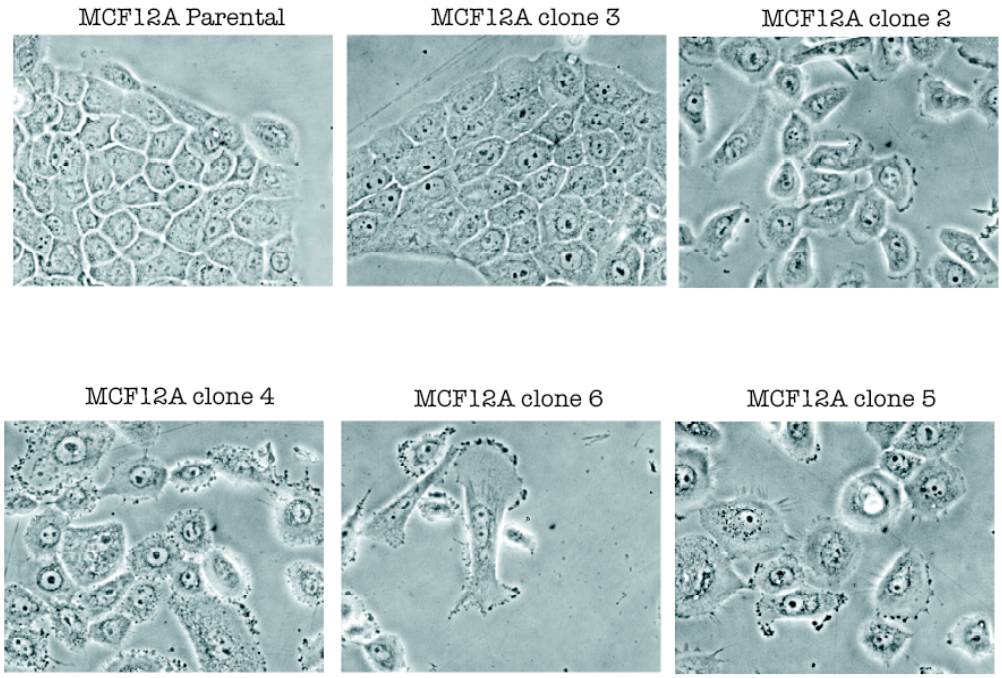


Figure 15. Effects of forced WT ROR1 overexpression on the morphology of immortalized human mammary epithelial cells. MCF12A cells were transfected with either ROR1 or a vector control, cultures were selected with Zeocin and individual colonies were isolated and expanded. A) Bright field images at 10X. B) Bright field images at 32X.

Key Research Accomplishments

- Validated that ROR1 is expressed exclusively in non-luminal breast cells in a large panel of 51 breast cell lines.

- Identified ROR1-expressing, non-luminal breast cell lines with multi-potent potential (i.e. CAL51, 184A1).

- Generated several retroviral constructs that express small interfering RNA's (hpRNA's) to silence ROR1 expression along with negative control constructs.

- Generated stable viral packaging cell lines expressing each of the hpRNA constructs.

- Generated stable breast cell lines expressing hpRNA for ROR1 and negative controls.

- Generated a panel of ROR1 mammalian expression constructs including a series of cytoplasmic point mutations to probe structure-function relationships.

- Determined that silencing of ROR1 in breast cancer cells leads to a phenotype in 3D matrigel culture and in tumor xenografts consistent with a block in differentiation potential.

- Identified a strong developmental genetic program that regulates histogenesis (especially bone and muscle formation) that is associated with ROR1 depletion.

- Uncovered a potential role for ROR1 in the inhibition of the Hedgehog pathway. This molecular interaction could help explain why loss of ROR1 in CAL51 cells leads to less differentiated phenotype in the presence of increased Hedgehog signaling which has been described to be important in the maintenance of adult progenitor cell populations.

- Identified a reproducible "migratory" phenotype associated with overexpression of WT ROR1 in human mammary epithelial cells.

Reportable Outcomes

1) Research Reagents: Constructs and cell lines:

Table 1. Mammalian expression constructs

Description	
pSiren-RetroQ vector containing a hairpining defective Luciferase control oligo.	Plasmid DNA
pSiren-RetroQ vector containing a siRNA for Luciferase.	Plasmid DNA
pSiren-RetroQ vector containing a siRNA for ROR1 (seq #9)	Plasmid DNA
pSiren-RetroQ vector containing a siRNA for ROR1 (seq #16)	Plasmid DNA
pcDNA4/TO-ROR1 (WT): Tet inducible expression plasmid	Plasmid DNA
pcDNA4/TO-ROR1 (D615A): Mutant (tet inducible)	Plasmid DNA
pcDNA4/TO-ROR1 (K506A): Mutant (tet inducible)	Plasmid DNA
pcDNA4/TO-ROR1 (Y641W): Mutant (tet inducible)	Plasmid DNA
pcDNA4/TO-ROR1 (Y722W): Mutant (tet inducible)	Plasmid DNA
pcDNA3.1-ROR1 Constitutive ROR1 expression	Plasmid DNA

Table 2. Cells lines isolated with stable expression of hpROR1 silencing constructs or control silencing sequences.

Parental Line	Description	ROR1 (array) log ratio	ROR1 (North) log ratio	siRNA
CAL51	Basal-Mesenchymal	0.60	0.82	Neg
CAL51	"			Luc
CAL51	"			#9
CAL51	"			#16
MDA-MB-468	Basal	0.60	0.65	Neg
MDA-MB-468	"			Luc
MDA-MB-468	"			#9
MDA-MB-468	"			#16
HCC1187	Basal	0.90	1.04	Neg
HCC1187	"			Luc
HCC1187	"			#9
HCC1187	"			#16
MCF12A	Basal (non-malignant)	0.04	-0.19	Neg
MCF12A	"			Luc
MCF12A	"			#9
MCF12A	"			#16
Hs578T	Mesenchymal	0.41	0.46	Neg
Hs578T	"			Luc
Hs578T	"			#9
Hs578T	"			#16
MDA-MB-231	Mesenchymal	0.50	0.56	Neg
MDA-MB-231	"			Luc
MDA-MB-231	"			#9
MDA-MB-231	"			#16
HCC1395	Mesenchymal	0.49	0.55	Neg
HCC1395	"			Luc
HCC1395	"			#9
HCC1395	"			#16

Patent Applications:

United States Patent Application: 20050079508 April 14, 2005
Dering, Judy; *Wilson, Cindy A.* Slamon, Dennis;
"Constraints-based analysis of gene expression data"

Patent Application claiming priority to U.S. Provisional Patent Application Serial No. 60/559,762
Inventor(s): Cindy A. Wilson, and Judy Dering, Dennis J. Slamon,
"ORPHAN RECEPTOR TYROSINE KINASE AS A TARGET IN BREAST CANCER".

Presentations:

July 2004	Presentation: Fourth International Symposium on Translational Research in Oncology: "Breast Cancer Classification: Biological and Therapeutic Implications". Dublin, Ireland.
June 2005	Symposium Presentation 2005 Era of Hope Meeting: "Stratification of Human Breast Cancer by mRNA expression profiling". Philadelphia, Pennsylvania.

Abstracts and Posters:

Wilson, C. A., Dering J., Bernardo G., Rong HM., Ginther, C., Ferdman R., Cook AM., Finn R., and Slamon, D. J. Cell Differentiation and Dominant Signaling Pathway Signatures in the Molecular Classification of Human Breast Cancer Cell Lines. *Third International Symposium on the Molecular Biology of Breast Cancer.* Molde, Norway (2005).

Wilson, C. A., and Dering., J. Role of the ROR1 Receptor Tyrosine Kinase in Basal, ER-Negative Breast Cancer. *Department of Defense Breast Cancer Research Program Meeting: 4th Era of Hope Meeting,* Philadelphia, PA. (2005).

Wilson, C. A., Bernardo, G., Rong, H. M., and Dering., J. An orphan receptor tyrosine kinase and the control of multi-potent mammary progenitor cells. *Department of Defense Breast Cancer Research Program Meeting: 4th Era of Hope Meeting*. Philadelphia, PA. (2005).

Training and Employment:

Gina Bernardo, research associate, was supported in part by this grant and her experiences on this project in large part led her to continue breast cancer research in graduate school in the Pharmacology Department of Case Western Reserve in the laboratory of Dr. Keri where she was awarded her own Department of Defense Breast Cancer Research Program (BCRP) Pre-doctoral Traineeship Award, 2006-2009.

Cindy Wilson, began a research collaboration with Amgen, Inc. Thousand Oaks, CA based primarily on the work funded by this grant. The constructs and cell lines listed above were licensed to Amgen to further this research partnership. This collaboration led to my recruitment and eventual employment with Amgen at the site in Cambridge, MA.

Conclusion:

ROR1 expression is detected only in breast cells that have non-luminal characteristics. Depletion of ROR1 in breast cells by siRNA induces phenotypic changes and altered growth patterns consistent with a loss of a differentiation potential. ROR1 silencing is also associated with a striking change in a developmental genetic program regulating histogenesis. The potential interactions between ROR1, which has recently been reported to be a Wnt receptor in the planar cell polarity pathway, and the Hedgehog pathway need to be followed up with additional experiments. There is also much biology that needs to be understood about consequences of either agonizing or antagonizing the ROR1 receptor. However, if these interactions between these crucial developmental pathways (Wnt and Hedgehog) can be elucidated and if the role of ROR1 in the growth and/or maintenance of mammary progenitor cells can be confirmed in additional models, its properties as both cell surface receptor and a tyrosine kinase make it an attractive therapeutic target.

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